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<b>(21) International Application Number:</b> PCT/US96/06076 <b>(22) International Filing Date:</b> 30 April 1996 (30.04.96) <b>(30) Priority Data:</b> 08/435,040 8 May 1995 (08.05.95) US <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). <b>(72) Inventors:</b> HAMMOCK, Bruce, D.; 3134 Chesapeake Bay Avenue, Davis, CA 95616 (US). HERRMANN, Rafael; 31 Hagefen Street, 27000 Kiryat Bialic (IL). MOSKOWITZ, Haim; Apartment 7, 15 Guatemala Street, 96704 Jerusalem (IL). <b>(74) Agents:</b> SIEBERT, J., Suzanne et al.; Majestic, Parsons, Siebert & Hsue, Suite 1450, Four Embarcadero Center, San Francisco, CA 94111-4121 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> INSECT CONTROL WITH MULTIPLE TOXINS  <b>(57) Abstract</b> <p>A method is provided that accelerates the rate of kill of pests such as from the order <i>Lepidoptera</i>. The method comprises treating the pests or their loci with at least two different insect toxins which are expressed from at least one recombinant microbe. Pairs of toxins that do not compete with each other on the same binding site and that differ in their pharmacology have been found to provide synergistic control. Preferred insecticidal microbes are baculoviruses.</p>		

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## INSECT CONTROL WITH MULTIPLE TOXINS

5    Field of the Invention

          The present invention generally relates to  
uses of insect toxins in controlling insects, and more  
particularly to insecticidal recombinant microbes  
expressing insect-selective toxins in synergistic  
10 combinations to magnify insect kill rate.

          This invention was made with government  
support under Grant No. 91-37302-6185, awarded by the  
United States Department of Agriculture. The U.S.  
Government has certain rights in the invention.

15   Background of the Invention

          The lepidopteran family Noctuidae includes  
some of the most destructive agricultural pests, such as  
the genera *Heliothis*, *Helicoverpa*, *Spodoptera*, and  
*Trichoplusia*. For example, included in this family are  
20 the tobacco budworm (*Heliothis virescens*), the cotton  
bollworm (*Helicoverpa zea*), the cotton leafworm (*Alabama*  
*argillacea*), the spotted cutworm (*Amathes niarum*), the  
glassy cutworm (*Crymodes devastator*), the bronzed  
cutworm (*Nephelodes emmedonia*), the fall armyworm  
25 (*Laphygma frugiperda*), the beet armyworm (*Spodoptera*  
*exigua*), and the variegated cutworm (*Peridroma saucia*).

          Resistance of agricultural pests, such as the  
*Noctuidae* (and others), to pesticides leads to  
environmental and human health risks. This problem of

insecticide resistance leads to the use of more non-selective and toxic compounds, in order to overcome pest resistance. This creates a destructive and vicious cycle.

5            Selective natural toxins have been suggested for use in insect control. These toxins include substances which are produced in specialized glandular tissues in the body of a venomous animal. The venom may be introduced into the body of its prey or opponent, 10 such as by the aid of a stinging-piercing apparatus, in order to paralyze and/or kill it, although other means of delivering venom are known. Scorpions, for example, contain in their venom a number of proteins, or neurotoxins, which are toxic and act on the excitable 15 systems. Among the insect specific toxins suggested for use in insect control are toxins from *Bacillus thuringiensis* from the scorpions *Buthus eupeus* and *Androctonus australis*, *Leiurus quinquestriatus hebraeus*, *Leiurus quinquestriatus quinquestriatus*, and from the mite *Pyemotes tritici*. 20

          The venoms derived from scorpions belonging to the Buthinae subfamily have four main groups of polypeptide neurotoxins which modify axonal sodium conductance. One group of scorpion neurotoxins are the 25  $\alpha$ -toxins, which selectively affect mammals through an extreme prolongation of the action potentials due to a slowing or blockage of the sodium channel inactivation (Catterall, *Science*, 223:653-661 (1984); Rochat et al., *Advances in Cytopharmacology*, pp. 325-334 (1979)). The 30 second group of toxins, the  $\beta$ -toxins, affect sodium channel activation (Couraud and Jover in *Handbook of Natural Toxins* (Tu, A. Ed.) Vol. 2, pp. 659-678 (1984) New York: Marcel Dekker. The third group of neurotoxins are the depressant insect selective toxins 35 which induce a progressively developing flaccid

paralysis of insects by the blockage of action potentials substantially due to the suppression of sodium current (Lester et al., *Biochim. Biophys. Acta*, 701:370-381 (1982); Zlotkin et al., *Arch. Biochem. Biophys.*, 240:877-887 (1985)). The fourth group of neurotoxins are the excitatory insect selective toxins which cause an immediate (knock down) spastic paralysis of insects by the induction of repetitive firing in their motor nerves due to an increase of the sodium peak current and the voltage dependent slowing of its inactivation (Walther et al., *J. Insect Physiol.*, 22:1187-1194 (1976); Pelhate et al., *J. Physiol.*, 30:318-319 (1981)).

In addition to scorpion and mite toxins, other insect-selective toxins have been identified in venoms from snails, spiders, and a number of other arthropods. [See review by Zlotkin, *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 10, Chapter 15, pp. 499-541 (1985).] The venoms of braconid wasps are highly toxic to lepidopterous larvae. The venom of the braconid *Bracon hebetor* causes a flaccid paralysis in lepidopterous larvae by inducing presynaptic interruption of the excitatory glutaminergic transmission at the insect neuromuscular junction (Piek et al., *Comp. Biochem. Physiol.*, 72C:303-309 (1982)). The venoms of solitary wasps are toxic to a large number of insects and spiders from different orders (Rathmeyer, *Z. Vergl. Physiol.*, 45:453-462 (1962)). An example of these venoms is the venom of *Philanthus triangulum* which induces in insects a flaccid paralysis substantially due to presynaptic blockage of neuromuscular transmission; this venom affects both excitatory and inhibitory transmission (May et al., *Insect Physiol.*, 25:285-691 (1979)). The venom of the black widow spider, *Latrodectus mactans*, contains components which are

neurotoxic to insects, but not to mammals, and other components with the opposite selectivity (Fritz et al., *Nature*, 238:486-487 (1980); Ornberg et al., *Toxicon*, 14:329-333 (1976)).

5 More recently, a toxin designated as LqhQIT, which strongly reassembles  $\alpha$  toxins in its primary structure and electrophysiological effects, was isolated from the venom *L. quinquestriatus hebraeus* and was shown to affect mainly insects (Eitan et al., *Biochemistry*, 29  
10 (1990), pp. 5941-5947).

The venom of venomous animals is composed of a variety of toxins affecting different target sites in the excitable systems of the prey. On the basis of the data comparing the activity of toxins and their  
15 respective crude venom towards lepidopterous larvae it is clear that the potency of the crude venom cannot be explained by the activity of one toxin alone. The higher potency of the crude venom could be related to a cooperativity among different toxins in the venom  
20 affecting different target sites on the same ionic channels (Table 3, Trainer et al., *JBC*, 268, 17114-17119 (1993)), different ionic channels on the same excitable cells (Olivera et al., *Science*, 249, 257-263 (1990)),  
and/or different binding sites on adjacent excitable  
25 cells (nerves and/or muscles) (Olivera et al., *Science*, 249, 257-263 (1990)).

The depressant and the excitatory insect-selective toxins do not compete with the  $\alpha$  insect toxin for its binding site (Gordon and Zlotkin, *FEBS Lett.*,  
30 315 (1993), pp. 125-128). In contrast to locust or cockroach neuronal membranes the excitatory toxins do not displace the depressant toxins from their binding sites in neuronal membranes of lepidopterous larvae (Gordon et al., *Biochemistry*, 31 (1992), pp. 76-22-7628;

Moskowitz et al., *Insect Biochem. Molec. Biol.*, 24 (1994), pp. 13-19).

Recently, the nuclear polyhedrosis virus *Autographa californica* (AcNPV), from the family Baculoviridae, has been genetically modified for an increased speed of kill by expressing insect-selective toxins. The introduction of an insect-selective toxin into an insect-pathogenic virus has resulted in a reduction in the killing time of insect hosts, as is described by U.S. Serial No. 08/229,417, filed April 15, 1994, which is a continuation-in-part application of U.S. Serial No. 07/629,603, filed December 19, 1990, having (in part) common assignment herewith.

Tomalski et al., U.S. Patent 5,266,317, issued November 30, 1993, discuss use of recombinant baculoviruses that express an insect-specific paralytic neurotoxin of an insect predacious mite. Barton et al., U.S. Patent 5,177,308, issued January 5, 1993, take a different approach in creating transgenic plants that express a scorpion derived insect-specific toxin and/or a soil dwelling microorganism toxin. In a copending application, of common assignment herewith, Hammock and McCutchen, Serial Number 08/279,956, filed July 5, 1994, discuss insect control with a synergistic combination of recombinant virus and an organic insecticide.

These newly emerging tools using recombinant strategies to control insect pest populations hold promise particularly since the wide-scale presence of pest resistance to organic insecticides, such as pyrethroids, has begun to result in substantial crop losses. In cotton alone, the presence of pyr-R *Heliothis* species has begun to result in millions of lost dollars annually. In fact, in several cases pyrethroid insecticides have completely failed to

control infestations of *Heliothis* larvae in cotton, which has resulted in complete destruction of the crop.

#### Summary of the Invention

In one aspect of the present invention, a method for controlling a variety of pests is provided through use of genetically engineered, insecticidal microbes. Pests controlled in accordance with the invention are, for example, from the group insects, acarids, and nematodes. Thus, the invention is applicable to the *Lepidoptera* as well as other orders, and to the *Noctuidae* as well as other families. Such pests are treated (or their loci treated) with a synergistic combination of toxins expressed by one or more recombinant microbes.

For example, the method may use a combination of first recombinant pathogen that expresses a first neurotoxin and a second recombinant pathogen that expresses a second neurotoxin, or may use a single recombinant virus expressing a plurality (such as the first and second) of neurotoxins. The inventive method accelerates the rate of kill of pests by the virus.

#### Brief Description of Drawing

In the drawing, Figure 1 illustrates the nucleotide sequence of a synthetic gene of LqhIV, SEQ ID NO:1, which one of the preferred toxins for practicing the invention.

#### Detailed Description of the Preferred Embodiments

The present invention is the use of genetically engineered, insecticidal microbes in combinations to treat pests such as insects. Although recombinant baculoviruses will be used throughout as an illustration of preferred microbes, this invention can



be practiced with a variety of microbes as recombinant delivery systems. Thus the microbes useful in the present invention include DNA and RNA viruses, such as baculoviruses, fungi and bacteria.

5           On the order of forty nuclear polyhedrosis viruses have been isolated from insect species. (See, for example, *Atlas of Invertebrate Viruses*, Adams and Bonami, editors, CRC Press, Inc., 1991.) Various baculoviruses, including those that infect cotton  
10 bollworm, *Helicoverpa zea*, tobacco budworm, *Heliothis virescens*, Douglas fir tussock moth, *Orgia pseudotsugata*, gypsy moth, *Lymantria dispar*, alfalfa looper, *Autographa californica*, European pine fly, *Neodiprion sertifer*, and codling moth, *Laspeyresia*  
15 *pomonella*, have been registered as pesticides and all such baculoviruses from insect species are suitable for practicing the invention.

Numerous fungi are capable of infecting insects. Introduction of the insect-selective toxin  
20 into the genome of such fungi could enhance the potency as pesticides. For example, *Beauveria bassiana* and *Beauveria brongniartii* have a wide host range and have been suggested as candidates for microbial pesticides (see review by Miller et al., *Science*, 219:715-721,  
25 1983).

Bacteria (other than *Bacillus thuringiensis*) that have been considered as insect control agents include *Bacillus popilliae*, *B. lentimorbus*, and *B. sphaericus*. Their potential as pesticides can be  
30 enhanced by improving their potency through the incorporation of an insect-selective toxin into their genome.

Practice of the invention involves the combined use of two, synergistically acting toxins in  
35 controlling insects. These two can be expressed by

means of a single recombinant microbe in which both toxin genes have been introduced, or may be practiced by preparing two recombinant microbes, each of which has been constructed by cloning a gene encoding the respective insect toxins into the genome. The combinations of toxin pairs selected are determined in several ways. As will be described hereinafter (as is described by the screening techniques of Example 6), one preferably selects toxins that act at the same cellular channels (typically sodium channels) but at non-overlapping sites, as will be further described hereinafter.

As earlier mentioned, preferred insecticidal microbes for practicing the invention are baculoviruses. By "baculovirus" is meant any baculovirus of the family *Baculoviridae*, such as a nuclear polyhedrosis virus (NPV). Baculoviruses are a large group of evolutionarily related viruses, which infect only arthropods; indeed, some baculoviruses only infect insects that are pests of commercially important agricultural and forestry crops, while others are known that specifically infect other insect pests. Because baculoviruses infect only arthropods, they pose little or no risk to humans, plants, or the environment.

Of the suitable DNA viruses, in addition to the *Baculoviridae* are the entomopox viruses (EPV), such as *Melolontha melonotha* EPV, *Amsacta moorei* EPV, *Locusta migratoria* EPV, *Melanoplus sanguinipes* EPV, *Schistocerca gregaria* EPV, *Aedes aegypti* EPV, and *Chironomus luridus* EPV. Other suitable DNA viruses are granulosis viruses (GV). Suitable RNA viruses include togaviruses, flaviviruses, picornaviruses, cytoplasmic polyhedrosis viruses (CPV), and the like. The subfamily of double stranded DNA viruses *Eubaculovirinae* includes two genera, NPVs and GVs, which are particularly useful

for biological control because they produce occlusion bodies in their life cycle. Examples of GVs include *Cydia pomonella* GV (coddling moth GV), *Pieris brassicae* GV, *Trichoplusia ni* GV, *Artogeia rapae* GV, and *Plodia interpunctella* GV (Indian meal moth).

Suitable baculoviruses for practicing this invention may be occluded or non-occluded. The nuclear polyhedrosis viruses ("NPV") are one baculovirus subgroup, which are "occluded." That is, a characteristic feature of the NPV group is that many virions are embedded in a crystalline protein matrix referred to as an "occlusion body." Examples of NPVs include *Lymantria dispar* NPV (gypsy moth NPV), *Autographa californica* MNPV, *Anagrapha falcifera* NPV (celery looper NPV), *Spodoptera littoralis* NPV, *Spodoptera frugiperda* NPV, *Heliothis armigera* NPV, *Mamestra brassicae* NPV, *Choristoneura fumiferana* NPV, *Trichoplusia ni* NPV, *Helicoverpa zea* NPV, and *Rachiplusia ou* NPV. For field use occluded viruses often are preferable due to their greater stability since the viral polyhedrin coat provides protection for the enclosed infectious nucleocapsids.

Among illustrative, useful baculoviruses in practicing this invention are those *Anagrapha falcifera*, *Anticarsia gemmatilis*, *Buzura suppressaria*, *Cydia pomonella*, *Helicoverpa zea*, *Heliothis armigera*, *Manestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, and *Spodoptera litura*. A particularly useful "NPV" baculovirus for practicing this invention is AcNPV, which is a nuclear polyhedrosis virus from *Autographa californica*. *Autographa californica* is of particular interest because various major pest species within the genera *Spodoptera*, *Trichoplusia*, and *Heliothis* are susceptible to this virus.

The expressed insecticidal toxins are particularly a neurotoxin derived from or similar to an arthropod or other invertebrate toxin, such as a scorpion toxin, a wasp toxin, a snail toxin, a mite toxin, or a spider toxin. A useful scorpion toxin is, for example, AaIT from *Androctonus australis*. Zlotkin et al., *Biochimie*, 53, 1073-1078 (1971). A useful snail venom is that from the snail *Conus querciones*, which the animal delivers by mouth and some individual toxins of which appear to be selective for arthropods including insects. See, for example, Olivera et al., "Diversity of *Conus* Neuropeptides," *Science*, 249:257-263 (1990).

Even peptides that normally appear in an insect's developmental life can operate as an insecticidal toxin, and be used in accordance with this invention. For example, the precocious appearance of juvenile hormone esterase ("JHE") will reduce juvenile hormone titers in a host insect, which typically results in irreversible termination of the feeding stage, attempted pupation, and death of the pest insect. The amino acid sequence of JHE is known, and the gene has been cloned. Preferred embodiments of the present invention include recombinant microbes expressing juvenile hormone esterase (JHE) mutations, and exemplary methods for preparing such JHE mutations or deletions, several useful JHE mutations, and recombinant expression vectors for use in controlling insects (having JHE or mutated JHE coding sequences) as are described by WO 94/03588, published February 17, 1994, inventors Hammock et al., incorporated herein by reference.

Two mutants described in the Hammock et al. WO 94/03588 are a double lysine mutant (K29R, K522R) where the normal lysines of JHE at position 29 and position 522 were changed to arginine by site-directed mutagenesis. Another mutant described was where serine

201 was changed to glycine and the mutant designated "S201G." The insecticidal activity of the catalytically deficient S201G mutant of JHE provided similar time for 50% death of test insects to scorpion toxins (when engineered in AcNPV). Thus, the naturally occurring JHE insect protein, which is not normally toxic, can be modified by means such as site-directed mutagenesis (or otherwise) to a toxic agent. In addition to amino acid residue changes, other JHE mutants could be prepared such as by deleting the N-terminal 19 amino acids, which are a signal sequence for the newly made protein to enter the secretory pathway, become glycosolated, and exit the cell.

As with JHE, the amino acid sequence of the excitatory toxin from *Androctonus australis* (AaIT) has also been determined, the sequence has been published (Darbon 1982), and the AaIT gene has been cloned and inserted into expression vectors for insect control. (See WO 92/11363, published July 9, 1992, inventors Belagaje et al.) The AaIT toxin exhibits toxicity to insects, while being non-toxic to isopods and mammals.

Yet another suitable toxin for practicing the invention affects insect sodium channels in a manner very similar to the effect of  $\alpha$ -toxins on mammalian sodium channels. This neurotoxin was derived from a yellow scorpion *Leirus quinquestriatus hebraeus*, and is called herein Lqh $\alpha$ IT. The identification and purification of this toxin was described in "A Scorpion Venom Neurotoxin Paralytic to Insects that Affects Sodium Current Inactivation: Purification, Primary Structure, and Mode of Action," published by Eitan et al., *Biochemistry*, 29:5941-5947 (1990).

Two preferred toxins for practicing the invention are novel in isolated and purified form, and will be described more fully hereinafter. Briefly,

these two are designated "LqhIV" and "LqhVI." These two toxins occur in the venom of *L. quingestriatus hebraeus*, which contains a number of individual toxins in admixture in the native form. The LqhIV toxin is an extremely potent lepidopterous toxin, shows positive cooperativity with other scorpion toxins when injected into the Lepidopterous larvae, and has no or weak mammal toxicity. A synthetic gene for this LqhIV toxin is illustrated by Fig. 1, SEQ ID NO:1.

Thus, the genes for these two preferred toxins can be synthesized (since the peptide sequence sizes are sufficiently small so as to make feasible synthesizing the DNA). Alternatively, the genes can be cloned. The coding sequences may then be cloned into a transfer vector, as will be exemplified further hereinafter.

We have demonstrated aspects of the invention with the synergistic combination of the toxins AaIT and LqhQIT in both blow fly larvae and in *Heliothis* larvae where the insecticidal activity of these insect-selective neurotoxins was increased five to ten fold when used in combination. Other combinations illustrating the invention and experimental details will be more fully discussed hereinafter.

Various other scorpion toxins (e.g. the Buthoid scorpion) can also be used for the synergistic combinations, such as LqqIT2, which is a depressive insect toxin from *Leiurus quinquestriatus quinquestriatus*. The purification method used to obtain this neurotoxin was published by Zlotkin et al., *Archives of Biochem. Biophys.*, 240:877-887 (1985).

BjIT2 is another depressive insect toxin and is from *Buthotus judaicus*. The purification has been published in Lester et al., *Biochim. Biophys. Acta*, 701:370-381 (1982). BjIT2 exists in two isoforms which

differ in amino acid sequence at position 15. Form 1 has isoleucine in this position while form 2 has valine.

LqhIT2 is yet another depressive insect toxin from *Leiurus quinquestriatus hebraeus* which was purified using reverse phase HPLC.

Yet other toxins, purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*, can also be used. For example, SmpIT2, from the chactoid scorpion, *Scorpio maurus palmatus*, is a depressive insect toxin. Its purification is described in Lazarovici et al., *J. Biol. Chem.*, 257:8397-8404 (1982).

Still other toxins purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*, are SmpCT2 and SmpCT3, and crustacean toxins, whose purification has been described in Lazarovici, Ph.D. thesis (1980), Hebrew University, Jerusalem, "Studies on the Composition and Action of the Venom of the Scorpion *Scorpio maurus palmatus* (Scorpionidae)."

Table 1 lists some preferred toxins for practicing this invention along with citations to their purification and characterization.

TABLE 1

	<u>ILLUSTRATIVE TOXINS</u>	<u>REFERENCES</u>
25	AaIT	Zlotkin et al., <i>Biochim</i> , 53, 1075-1078 (1971).
	AaIT <sub>1</sub>	Loret et al., <i>Biochem.</i> , 29, 1492-1501 (1990).
	AaIT <sub>2</sub>	Loret et al., <i>Biochem.</i> , 29, 1492-1501 (1990).
	LqqIT <sub>1</sub>	Zlotkin et al., <i>Arch. f Biochem. &amp; Biophys.</i> , 240, 877-887 (1985).
	BjIT <sub>1</sub>	Lester et al., <i>Biochem. Biophys. Acta</i> , 701, 370-387 (1982).

- LqhIT<sub>2</sub> Zlotkin et al., *Biochem.*, 30, 4814-4821 (1991).
- LqqIT<sub>2</sub> Zlotkin et al., *Arch. f Biochem. & Biophys.*, 240, 877-887 (1985).
- BjIT<sub>2</sub> Lester et al., *Biochem. Biophys. Acta*, 701, 370-387 (1982).
- LqhQIT Eitan et al., *Biochem.*, 29, 5941-5947 (1990).
- 5 TS<sub>VII</sub> Bechis et al., *Biochem. Biophys. Res. Comm.*, 122, 1146-1153 (1984).
- Mite toxin Tomalski et al., *Toxicon*, 27, 1151-1167 (1989).
- α-conotoxins Gray et al., *JBC*, 256, 4734-4740 (1981); Gray et al., *Biochem.*, 23, 2796-2802 (1984).
- μ-conotoxins Cruz et al., *JBC*, 260, 9280-9288 (1989); Crus et al., *Biochem.*, 28, 3437-3442 (1989).
- chlorotoxin Debin et al., *Am. J. Physiol.*, 264, 361-369 (1993).
- 10 ω-conotoxins Olivera et al., *Biochem.*, 23, 5087-5090 (1984); Rivier et al., *JBC*, 262, 1194-1198 (1987).
- PLTX1 Branton et al., *Soc. Neurosci. Abs.*, 12, 176 (1986).
- PLTX2 Branton et al., *Soc. Neurosci. Abs.*, 12, 176 (1986).
- PLTX3 Branton et al., *Soc. Neurosci. Abs.*, 12, 176 (1986).
- Ag1 Kerr et al., *Soc. Neurosci. Abs.*, 13, 182 (1987); Sugimori et al., *Soc. Neurosci. Abs.*, 13, 228 (1987).
- 15 Ag2 Kerr et al., *Soc. Neurosci. Abs.*, 13, 182 (1987); Jackson et al., *Soc. Neurosci. Abs.*, 13, 1078 (1987).
- ω-Agatoxin Adams et al., *JBC*, 265, 861-867 (1990).



$\mu$ -Agatoxin	Adams et al., JBC, 265, 861-867 (1990).
Hol	Bowers et al., PNAS, 84, 3506-3510 (1987).
$\alpha$ -Laterotoxin	Grasso, in Neurotoxins in Neurochemistry, ed. Dolly, 67-79 (1988).
Steatoda toxin	Cavalieri et al., Toxicon, 25, 965-974 (1987).
5 Bom III	Vargas et al., Eur. J. Biochem., 162, 589-599 (1987).

CDNA libraries for many of the organisms from which the Table 1 illustrative toxins can be purified are available as described by: Zilberberg et al. (1992), Insect Biochem. Molec. Biol, 22(2), 199-203 (1992), *Leiurus quinquestriatus hebraeus*); Gurevitz et al. (1990) Febs Lett., 269(1), 229-332 (*Buthus judaicus*); Bougis et al. (1989), JBC, 264(32), 19259-19256 (*Androctonus australis*); Martin-Euclaire et al. (1992) Febs. Lett., 302(3), 220-222 (*Tityus serrulatus*); 15 Woodward et al. (1990) EMBO J., 9(4), 1015-1020 (*Conus textile*); and Colledge et al. (1992) Toxicon, 30(9), 1111-11116 (*Conus geographus*). For others, one may construct synthetic genes coding for the toxins, in a manner analogous to that exemplified by Example 7.

20 As earlier mentioned, two toxins suitable for use in practicing the present invention are novel in their isolated and purified form. One of these is designated "LqhIV," and has the amino acid sequence shown as SEQ ID NO:2: GVRDAYIADD KNCVYTCGAN SYCNTECTKN 25 GAESGYCQWF GKYGNACWCI KLPDKVPIRI PGKCR. The SEQ ID NO:2 sixty-five amino acid peptide is further described in Example 5.

Another novel toxin, designed "LqhVI," has the amino acid sequence given by SEQ ID NO:3: GVRDGYIAQP

ENCVYHCFPG SPGCDTLCKG DGASSGHC GF KEGHGLACWC NDLPDKVGII  
VEGEKCH. This sixty-seven amino acid peptide is also  
further described by Example 5.

The toxins, such as the preferred toxins  
5 listed in Table 1, or as SEQ ID NOS:2 and 3, may be  
selected to form synergistic combinations most easily by  
first making experimental combinations of toxins having  
different pharmacologies. For example, AaIT is an  
excitatory insect toxin while LqhIT<sub>2</sub> is a depressant  
10 toxin. By routine binding protocols (see, e.g. Gordon  
et al., *Biochim. Biophys. Acta*, 778, 349-358 (1984) for  
AaIT, BjIT<sub>1</sub>, and BjIT<sub>2</sub> with locust *Locusta migratoria*  
membrane vesicles), one screens for activity at the same  
channel but at non-overlapping sites for the particular  
15 insect of interest. This is because, as known to the  
art, there is a variability among various insect  
neuronal membranes. For example, several recent  
articles have reported that unlike locust or cockroach  
neuronal membranes, *Lepidopterous* larvae neuronal  
20 membranes can bind the depressant and excitatory insect  
toxins at the same time.

In the earlier noted example of a synergistic  
combination of AaIT and LqhOXIT, there was twice the  
synergistic potency for the combination towards blow fly  
25 larvae than to *Heliothis* larvae. In contrast, with the  
combination of AaIT and LqhIT<sub>2</sub>, there is a synergistic  
combination (potency of five times) when applied to  
*Heliothis* larvae, but no increased potency with respect  
to each toxin by itself when applied to blow fly larvae.  
30 These combinations of toxins can be used to enhance  
selectivity within insect groups.

For producing recombinant microbes, such as  
baculoviruses, for the purpose of controlling insects,  
a secretion signal sequence is preferably included.  
35 Secretion signal sequences may be derived from proteins

of bacteria, yeast, fungi, or higher eukaryotes, including both animals and plants (for examples, see Watson, *Nucl. Ac. Res.*, 12:5145-5164 (1984). More preferred are secretion signal sequences from proteins of insect origin, for example those of cecropin B from *Hyalophora cecropia* (van Hofsten et al., *PNAS*, 82:2240-2243 (1985)), and the eclosion hormone from *Manduca sexta* (Horodyski et al., *PNAS*, 86:8123-8127 (1989)). Also preferred are the secretion signal sequences naturally associated with scorpion toxins, which can be determined by the analysis of mRNA, cDNA, or genomic DNA. More preferred is the natural secretion signal sequence of AaIT (Bougis et al., *J. Biol. Chem.*, 264:19259-19265 (1989)).

The toxins of the recombinant microbes may be expressed as functional derivatives of the toxin. A "functional derivative" of the toxin is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the toxin. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. By a "fragment" of a molecule such as a toxin is meant to refer to any polypeptide subset of the molecule. A "variant" of a molecule such as a toxin is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof. A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules is not found in the other, or if the sequence

of amino acid residues is not identical. An "analog" of a molecule such as the toxin is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used  
5 herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule.

Such moieties may improve the molecule's solubility, absorption, biological half-life, etc.  
10 Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedures for coupling such moieties to a molecule are well known in the art.

Expression of the toxin (or toxins) will, in  
15 general, include a promoter region sufficient to direct the initiation of RNA synthesis. One baculovirus promoter gene is that coding for polyhedrin, since the polyhedrin protein is one of the most highly expressed eucaryotic genes known, although other promoter and  
20 hybrid promoter sequences may be used, for example, such as p10.

Recombinant baculoviruses expressing one toxin may be prepared by protocols now known to the art (e.g. Tomalski et al., U.S. Patent 5,266,317, exemplifying  
25 neurotoxins from the insect-parasitic mites; McCutchen et al., *Bio/Technology*, 9, 848-852 (1991) and Maeda et al., "Insecticidal Effects of an Insect-Specific Neurotoxin Expressed by a Recombinant Baculovirus," *Virology*, 184, 777-780 (1991), illustrating construction  
30 of a recombinant baculovirus expressing AaIT).

Preparation of a single baculovirus capable of expressing two different toxins may by protocols analogous to: Belayev and Roy, *Nucleic Acid Research*, 21:5, 1219-1223 (1993); Wang et al., *Gene*, 100, 131-137

(1991), with appropriate modifications. Example 1 illustrates such an analogous protocol.

#### EXAMPLE 1

Two insect toxin genes may be cloned into a transfer vector such as PacUW51P2 using standard molecular cloning techniques. This transfer vector is an *Autographa californica* (AcNPV) polyhedron locust-based vector that contains a copy of the AcNPV p10 promoter and SV40 transcription termination signals inserted in tandem, upstream of the polyhedron gene promoter, but in opposite orientation. This facilitates the assertion of one foreign gene coding region at a Bam HI site under the control of the polyhedron promoter, and a second foreign gene coding region at a BglII cloning site, under the control of the p10 promoter. Thus, the resulting recombinant virus expresses two foreign proteins. The recombinant AcNPV thus prepared may be isolated by propagating *Spodoptera frugiperda* cells (Sf21), which are co-transfected by calcium precipitation with the recombinant plasmid. Polyhedron-infected cells can be identified and collected post infection and the recombinant virus plaque purified by screening. Purification of the recombinant virus may be by standard protocols, with the resultant, pure recombinant propagated and stored, such as at 4°C and -80°C. Standard protocols are described, for example, in O'Reilly, Miller and Luckow, *Baculovirus Expression Vectors, A Laboratory Manual*.

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EXAMPLE 2

The activities of four different insect toxins towards two different insects and towards mice were determined (since one prefers to use insect toxins that have little or no effect on mammals). These were purified by established methods from the respective crude venoms. Their toxicity towards mice and larvae of blow fly and lepidoptera was determined according to the method of Reed and Muench (1938).

Table 2 shows the activity of the toxins towards insects and mice in terms of fifty percent end points (paralytic or lethal doses  $PU_{50}$ ,  $LD_{50}$  respectively). The  $PU_{50}$  values of the toxins to blow fly larvae were in accordance with previously published results (Zlotkin et al., *Biochim*, 53, 1075-1078 (1971); and Eitan et al., *Biochem.*, 29, 5941-5947 (1990)). The toxicity of those toxins towards the lepidopterous larvae of *Heliothis virescens* is comparable to their toxicity to larvae of *Spodoptera littoralis*. LqhOIT showed higher toxicity towards mice (Swiss Webster), but the other toxins showed no toxicity to mammals (3  $\mu$ g/g b.w injected subcutaneously had no effect, in contrast to the  $LD_{50}$  of a mammalian toxin AaH<sub>II</sub> - 0.018  $\mu$ g/20 g b.w (DeLima et al., 1986). The toxins LqhIV and LqhVI are of considerable interest since LqhIV is the most potent lepidopterous toxin isolated from scorpion venom to date while the toxin LqhVI has weak mammal toxicity.

TABLE 2

Toxins	PU <sub>50</sub> to <i>Sarcophaga falcitata</i> larvae (µg/100 mg b.w.) <sup>a</sup>	PU <sub>50</sub> to <i>Heliothis virescens</i> larvae (µg/100 mg b.w.) <sup>b</sup>	LD <sub>50</sub> to Swiss Webster mice (µg/20 g b.w.) <sup>c</sup>
AaIT	0.0025	2.5	>60
LqhIT <sub>3</sub>	0.050	2.5	>60
LqhIT <sub>2</sub>	0.025	2.5	>60
LqhαIT	0.0025	2.5	8.0
LqhIV	0.1	0.5	12
LqhVI	0.006	3.0	>60

- 10     <sup>a</sup> Three replicates of 25-40 blow fly larvae each were injected with each one of the toxins and the PU<sub>50</sub> were determined. The PU<sub>50</sub> of the excitatory toxins AaIT, LqhVI, and LqhIT<sub>3</sub> was determined as a contraction paralysis immediately after injection. The PU<sub>50</sub> of the depressant toxin LqhIT<sub>2</sub> was determined as a flaccid paralysis 5 minutes after injection. The PU<sub>50</sub> of the α insect toxins LqhαIT and LqhIV was determined as a delayed and sustained contraction paralysis 5 minutes after injection.
- 15     <sup>b</sup> Three replicates of 25-40 lepidopterous larvae each were injected with each one of the toxins and the PU<sub>50</sub> was determined as inability to move or turn when inverted on its back 24 hours after injection.
- <sup>c</sup> Two replicates of eight mice were injected subcutaneously and the LD<sub>50</sub> to mice was determined 24 hours after injection.

20

EXAMPLE 3

Combinations of toxins were injected simultaneously, and toxicity was measured as summarized in Table 3. The toxin combinations included amounts corresponding to 1 PU<sub>50</sub> unit of each toxin and their dilutions. Pairs of toxins that do not compete with each other on the same binding site and differ in their pharmacology were synergistic. As shown in Table 3, the degree of cooperatively is not only dependent on the toxin combinations but also on the test animal.

TABLE 3

Toxin	PU <sub>50</sub> to <i>Sarcophaga falcitata</i> larvae (µg/100 mg b.w.) <sup>a</sup>		PU <sub>50</sub> to <i>Heliothis virescens</i> larvae (µg/100 mg b.w.) <sup>b</sup>		LD <sub>50</sub> to Swiss Webster mice (µg/20 g b.w.) <sup>c</sup>	
	Dose	Change in Potency <sup>a</sup>	Dose	Change in Potency <sup>b</sup>	Dose	Change in Potency <sup>c</sup>
AalT + LqhIT <sub>2</sub>	0.0025 (AalT) 0.025 (LqhIT <sub>2</sub> )	0.5X	0.25 (AalT) + 0.25 (LqhIT <sub>2</sub> )	5X	60 (AalT) + 60 (LqhIT <sub>2</sub> )	No effect
AalT + LqhαIT	0.000125 (AalT) 0.000125 (LqhαIT)	10X	0.25 (AalT) + 0.25 (LqhαIT)	5X	60 (AalT) + 8.0 (LqhαIT)	8.0
LqhIT <sub>3</sub> + LqhIT <sub>2</sub>	Not determined		0.25 (LqhIT <sub>3</sub> ) + 0.25 (LqhIT <sub>2</sub> )	5X	60 (LqhIT <sub>3</sub> ) + 60 (LqhIT <sub>2</sub> )	No effect
LqhIT <sub>3</sub> + LqhαIT	0.005 (LqhIT <sub>3</sub> ) 0.00025 (LqhαIT)	5X	0.25 (LqhIT <sub>3</sub> ) + 0.25 (LqhαIT)	5X	60 (LqhIT <sub>3</sub> ) + 8.0 (LqhαIT)	8.0

- 15      \* Three replicates of 25-40 blow fly larvae each were injected with a combination of toxins and the PU<sub>50</sub> was determined as fast contraction of the larvae within one minute after injection.
- 20      <sup>b</sup> Three replicates of 25-40 lepidopterous larvae each were injected with a combination of toxins and the PU<sub>50</sub> was determined as inability to move or turn when inverted on its back. The PU<sub>50</sub> was determined 24 hours after injection.
- 25      <sup>c</sup> Two replicates of eight mice were injected subcutaneously and the LD<sub>50</sub> to mice was determined 24 hours after injection.
- 25      \* The potency was estimated as the amount of toxin protein (a 1:1 ratio of toxins in various dilutions were used) that caused an effect compared to the PU<sub>50</sub> of each toxin alone.

30      As illustrated by Table 3, the combinations with greater than one potency were dose-responses greater than potentiation. Thus, these combinations are synergizing the rate of kill and illustrate preferred embodiments of the invention.



EXAMPLE 4

In practicing the invention, pests being controlled are treated (and/or their loci treated) with recombinant baculoviruses expressing such combinations. In this example, the combined application of two viruses expressing two different toxins is shown to reduce the time to kill a host insect when compared to application of each respective virus by itself. Thus, as shown in Table 4, the combined application of the recombinant AcAaIT with the recombinant AcLqhQIT resulted in substantially reduced kill times.

TABLE 4

Recombinant Application	LT <sub>10</sub>	LT <sub>50</sub>	LT <sub>90</sub>
AcLqhQIT (alone)	62	73	87
AcAaIT (alone)	55	68	82
AcAaIT + AcLqhQIT (combined, inventive embodiment)	45	60	80

Lethal times (LT's) were derived based on the response of third instar *H. virescens* larvae to AcAaIT (10000 PIB's), AcLqhQIT (10000 PIB's) and a combined application of AcAaIT (5000 PIB's) and AcLqhQIT (5000 PIB's). Small plugs of diet were placed in individual wells of microtiter plates and inoculated with either of the respective viruses. Third instar larvae of *H. virescens* were then added to the plates and held at 27°C. Mortality was recorded at 5-10 intervals. LT's were analyzed with a Probit analysis program.

Thus, the data of Table 4 is a study of speed of kill expressed as lethal times (LTs) and analogous approaches can be used to determine lethal doses, which are likely to be of major economic importance. Taking,

for example, the lethal time at which 50% of the larvae were dead, one sees that the combination of toxins in practicing the inventive method provided approximately a 12% to 18% reduction in the time required to kill the host larvae with respect to applications of individual recombinants. When one considers that treatment with the recombinant AcAaIT represents an approximate 40% reduction in the time required to kill host larvae when compared to wild-type AcNPV, one sees a substantial decrease in insect feeding damage and significantly less damaged plants result from practice of the invention. Further, larvae infected with recombinant microbes typically start showing symptoms of paralysis and stop feeding a number of hours prior to death, which further increases the practical insecticidal effects of the inventive method.

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#### EXAMPLE 5

##### Purification of LqhIV and LqhVI

Venom of the scorpion *L. quingestriatus hebraeus* was obtained from Sigma (USA).

Lyophilized *L. quingestriatus hebraeus* venom (50 mg) was suspended and homogenized in 2 ml of 10 mM Ammonium acetate pH=6.4. The insoluble material removed by centrifugation at 27000g for 20 min. The supernatant was collected and the pellet was resuspended in additional 2 ml of 10 mM ammonium acetate pH=6.4, homogenized and centrifuged again. This extraction was done 4 times to maximize the yield of protein extracted from the venom. The supernatant from all the centrifugations was pooled, loaded on a cation exchanger column (10 ml of CM-52) and eluted with a linear gradient of 0.01-0.5 M ammonium acetate pH=6.4 in a flow

rate of 10 ml/hr. Absorbance was monitored at 280 nm and peaks collected accordingly. The fraction CM-III and CM-VI from the cation exchanger chromatography were further purified on RP-HPLC on Vydac C4 Column. LqhIV was purified from CM-VI as follows: buffer A was 5% ACN with 0.1% TFA and buffer B was 95% ACN with 0.1% TFA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-60% B in 70 min, the flow rate was 0.6 ml/min. Absorbance was monitored at 214nm and peaks collected accordingly LqhVI was purified from CM-III as follows: buffer A was 5% ACN with 0.1% HFBA and buffer B was 95% ACN with 0.1% HFBA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-90% B in 105 min, the flow rate was 0.6 ml/min. Absorbance was monitored at 214 nm and peaks collected accordingly. The eluted fractions were collected and tested for activity (Table 2) and purity.

#### Purity of toxins

The homogeneity and purity of LqhIV and LqhVI was tested by Free Solution Capillary Electrophoresis (Applied Biosystems Model 270A). The capillary was equilibrated with 20 mM Sodium citrate pH=2.9 and the samples (0.02 mg/ml protein) were loaded using vacuum for two seconds. The running buffer was 20 mM sodium citrate pH=2.9, the electric force was 20KV.

#### Sequence determination

20  $\mu$ g of each toxin were reduced and carboxymethylated using established method (Fernandez et al., "Techniques in Protein Chemistry," Vol. 5 page 215). The N-terminal sequence was determined using HP sequence analyzer by automated Edman degradation. Reduced and carboxymethylated LqhIV was digested using Endoproteinase Asp-N and peptides were generated.

Separation of the digested peptides was done on microbore HPLC (Ultrafast Microprotein analyzer- Michrom BioResources Inc) using polymeric column. Buffer A was 5% ACN with 0.1% TFA and buffer B was 95% ACN with 0.1% TFA. The column was equilibrated in buffer A and eluted with a linear gradient of 0-50% B in 50 min, the flow rate was 0.05 ml/min. Absorbance was monitored at 214 nm and peaks collected accordingly. Peptide P2 was sequenced in order to determine the full amino acid sequence of the toxin.

#### EXAMPLE 6

##### Binding Protocol

##### Preparation of Insect Neuronal Membranes

All dissections and preparations of insect neuronal tissues are performed in a cold buffer of the following composition: 0.25 M mannitol, 10 mM EDTA pH=7.4, 5 mM HEPES (adjusted to pH 7.4 with Tris), 50 µg/ml phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 1 mM iodoacetamide and 1 mM 1,10-phenantroline. Insect nervous tissues are dissected and homogenized in ice cold buffer, the debris is removed by centrifugation at 1,000 g for 10 min. The supernatant is centrifuged at 27,000g for 45 min and the membranes are collected (P<sub>2</sub>). The P<sub>2</sub> is suspended in the buffer and adjusted to 10% Ficoll (in the buffer) and centrifuged at 10,000 g for 75 min. The resulting floating pellicle representing the enriched synaptosomal fraction is collected. Following treatment by hypotonic medium (5 mM Tris-HCl pH=7.4, 1 mM EDTA, 50 µg/ml phenylmethylsulfonyl fluoride, 1µM pepstatin A, 1 mM iodoacetamide and 1 mM 1,10-phenantroline) membrane vesicles are formed. The membrane vesicles are collected in a small volume of

dissection buffer after centrifugation 27,000 g for 45 min and stored at -80°C until use.

#### Radioiodination of Toxins

The toxins are iodinated by iodogen (Pierce  
5 Chemical Co., Rockville, MD) using 0.5 mCi of carrier-free  $\text{Na}^{125}\text{I}$  (~0.3 nmol) (Amersham) and 5 mg (~0.7 nmol) of toxin. The monoiodotoxin is purified on HPLC using a Beckman Ultrapore C3 RPSC column (4.6x75 mm) fractions are eluted at gradient of 10-80% solvent B (solvent A =  
10 0.1% TFA, solvent B = 50% ACN, 50% 2-propanol and 0.1% TFA) at flow rate of 0.5 ml/min. The monoiodotoxin is eluted as the first peak of radioactive protein (about 30% solvent B) following the peak of the native toxins (about 28% solvent B). The concentration of the  
15 radiolabeled toxin is estimated according to the specific radioactivity of  $^{125}\text{I}$  and correspond to 2424 dpm/fmol monoiodotoxin.

#### Binding Assays

Competitive binding assays are performed at  
20 equilibrium conditions using increasing concentrations of an unlabeled toxin in the presence of a constant concentration of a labeled toxin. Analysis of all binding assays is performed using the iterative computer program LIGAND (P.J. Munson and D. Rodbard, modified by  
25 G.A. McPherson 1985). Insect membrane vesicles are suspended in binding medium containing 0.13 M choline chloride, 1 mM EDTA pH=7.4, 20 mM HEPES/Tris pH=7.4 and 5 mg/ml BSA. Following 1 hour incubation with the toxins, the reaction mixture is diluted with 2 ml ice-  
30 cold wash buffer (150 mM choline chloride, 5 mM HEPES/Tris pH=7.4, 1 mM EDTA pH=7.4 and 5 mg/ml BSA) and filtered over GF/F filters (Whatman, U.K.) under vacuum, followed by two more washes of the filters with 2 ml of

wash buffer each time. Non-specific toxin binding is determined in the presence of 1  $\mu$ M unlabeled toxin.

#### EXAMPLE 7

##### Construction of the Synthetic Gene (Fig. 1, SEQ ID NO:1)

5           The protein sequence of a toxin was converted into a nucleotide sequence using the preferred codon usage of a baculovirus. The toxin gene along with the nucleotide sequence of a leader sequence (bombyxin, native leader or other) and the appropriate restriction  
10       enzyme sites were used to design and synthesize 5 complementary pairs of oligonucleotides. The oligonucleotides were phosphorilated, annealed, ligated and amplified by PCR using the outside oligonucleotides as primers. The PCR product is blunt end ligated into  
15       a PCRscript plasmid and the correct sequence was confirmed by sequencing. A BamHI restriction fragment was rescued from this plasmid cloned into a baculovirus transfer vector under a baculovirus promoter (P10, polyhedrin, Basic, IE1 etc.). A plasmid containing the  
20       correct sequence of the gene and leader sequence was confirmed by sequencing. Using the resulting transfer vector and the standard procedures a recombinant virus expressing the toxin was constructed.

##### Construction of a Virus Expressing AaIT and LqhIV

25           The leader sequence of bombyxin and the gene coding for the toxin LqhIV were designed and synthesized as described above. The correct sequence was confirmed and the gene was cloned into a double expression transfer vector already containing the AaIT  
30       gene. The transfer vector pAcUW51P2 is a polyhedrin positive vector with two cloning sites, a BglII site and the LqhIV gene with the bombyxin leader were cloned into

the BamHI site. Sf21 cells were cotransfected with the resulting transfer vector and infectious virus particles using the lipofectin procedure. Recombinant viruses were selected as a polyhedrin positive phenotype in a standard plaque assay. Sf21 cells were inoculated with the recombinant virus AcAaLq according to standard procedures. Protein extracts from virus infected cells were separated on 15% SDS-PAGE gels and then electroeluted to nitrocellulose membranes. The membranes were probed with AaIT and LqhIV antibodies, bound antibodies were detected using rabbit IgG HRP conjugates.

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In conclusion, genetically engineered, insecticidal microbes are produced in accordance with the invention and then used to control a variety of pests. In doing so, one may use a single recombinant virus expressing a plurality of neurotoxins. The combination of toxins are determined by selecting toxins that act at the same cellular channels (typically sodium channels) but at non-overlapping sites. Alternatively, one may use two (or more) recombinant insecticidal microbes where each expresses a different toxin. Again, the several expressed toxins are selected as already described. These combinations of expressed toxins accelerate the rate of kill of pests by the virus or viruses beyond simply an "additive" function. For example, the lethality of toxins AaIT and LqhQIT in both blowfly larvae and in *Heliothis* larvae was increased 5-10 fold when used in combination. Further, the combinations of toxins can be used to enhance selectivity within insect groups.

Conventional application means of the recombinant microbes (spraying, atomizing, dusting, scattering, or pouring) may be used from formulations

such as powders, dusts, granulates, as well as encapsulations such as in polymer substances. Compositions will typically include inert carriers such as clay, lactose, defatted soy bean powder, and the like  
5 to assist in applications, in order to apply the recombinant microbes expressing synergistic combinations of insecticidal toxins.

It is to be understood that while the invention has been described above in conjunction with  
10 preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.



It Is Claimed:

1. A method for controlling pests from the group insects, acarids, and nematodes comprising:  
treating said pests or their loci with at least two different insect toxins, the source of the toxins being at least one recombinant microbe or being  
5 a plurality of recombinant microbes, the toxins having non-overlapping binding sites at an insect cellular membrane channel.
2. The method as in claim 1 wherein the source of the toxins are recombinant insect viruses.
3. The method as in claim 1 wherein the source of recombinant insect viruses are baculoviruses.
4. The method as in claim 3 wherein the baculoviruses are nuclear polyhedrosis viruses.
5. The method as in claim 3 wherein the baculoviruses are from *Autographa californica*, *Anagrapha falcifera*, *Anticarsia gemmatalis*, *Buzura suppressoria*, *Cydia pomonella*, *Helicoverpa zea*, *Heliothis arrigera*,  
5 *Mariestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, or *Spodoptera litura*.
6. The method as in claim 1 wherein the toxins are combinations of AaIT, LqhIT<sub>2</sub>, LqhCIT, and LqhIT, neurotoxins.
7. The method as in claim 1 wherein the toxins include a JHE mutant.

8. The method as in claim 6 or 7 wherein the pest is *Heliothis virescens* or blow fly.

9. A substantially pure insect toxin, useful in insect control, having the amino acid sequence SEQ. ID NO:1 or SEQ. ID NO:2.

10. A recombinant microbe which, in insect cells infected therewith, expresses at least two foreign proteins toxic to the insect cells, or functional derivatives thereof, the microbe genome provided with a  
5 secretion signal sequence.

11. The recombinant microbe as in claim 10 wherein the microbe is a nuclear polyhedrosis virus.

12. The recombinant microbe as in claim 10 or 11 wherein at least one of the foreign proteins is a scorpion toxin.

13. The recombinant microbe as in claim 10 wherein the foreign proteins are derived from genetic sequence codings of scorpion, wasp, snail, mite, or spider venoms.

14. The microbe as in claim 10, 12, or 13 wherein the baculovirus is *Autographa californica* nuclear polyhedrosis virus.

15. An insect control composition comprising:  
a first and a second recombinant baculovirus,  
the first and second baculoviruses respectively  
expressing a first and a second toxin, the first and  
5 second toxins having non-overlapping binding sites at an  
insect cellular membrane channel.

16. An insect control composition comprising:  
a recombinant baculovirus which, in insect  
cells infected therewith, expresses a plurality of  
insecticidal toxins.

AGA TCT GGA TCC ATG AAG ATC CTC CTT GCT ATT GCC CTT ATG CTT AGC ACC GTG  
TCT AGA CCT AGG TAC TTC TAG GAG GAA CGA TAA CGG GAA TAC GAA TCG TGG CAC

ATG TGG GTG AGC ACC GGC GTG CGC GAC GCC TAC ATC GCC GAC GAC AAG AAC TGC  
TAC ACC CAC TCG TGG CCG CAC GCG CTG CGG ATG TAG CCG CTG CTG TTC TTG ACG

GTG TAC ACC TGC GGC GCC AAC TCT TAC TGC AAC ACC GAC TGC ACC AAG AAC GGC  
CAC ATG TGG ACG CCG CGG TTG AGA ATG ACG TTG TGG CTC ACG TGG TTC TTG CCG

GCC GAC TCT GGC TAC TGC CAA TGG TTC GGC AAA TAC GGC AAC GCA TGC TGG TGC  
CGG CTC AGA CCG ATG ACG GTT ACC AAG CCG TTT ATG CCG TTG CGT ACG ACC ACG

ATC AAA CTT CCC GAC AAA GTG CCC ATC CGC ATT CCC GGC AAA TGC CGC TAA GGA  
TAG TTT GAA GGG CTG TTT CAC GGG TAG GCG TAA GGG CCG TTT ACG GCG ATT CCT

TCC AGA TCT GAG CTC  
AGG TCT AGA CTC GAG

Fig. 1

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(54) Title: INSECT CONTROL WITH MULTIPLE TOXINS

## (57) Abstract

A method is provided that accelerates the rate of kill of pests such as from the order *Lepidoptera*. The method comprises treating the pests or their loci with at least two different insect toxins which are expressed from at least one recombinant microbe. Pairs of toxins that do not compete with each other on the same binding site and that differ in their pharmacology have been found to provide synergistic control. Preferred insecticidal microbes are baculoviruses.

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International Application No  
PL./US 96/06076

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A01N63/02 C07K14/435 C12N15/86

### B. FIELDS SEARCHED

IPC 6 A01N C07K C12N

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>EP 0 374 753 A (CIBA-GEIGY AG) 27 June 1990</p> <p>see page 2, line 1 - line 32</p> <p>see page 4, lines 38-41, 49-51</p> <p>see page 4, line 55 - page 6, line 12</p> <p>see page 14, line 46 - page 15, line 6</p> <p>see page 18, line 10 - line 31</p> <p>see page 19, line 57 - page 20, line 24;</p> <p>claims 1,27-34,38</p> <p>---</p>	<p>1-8, 10-16</p>
Y	<p>EP 0 431 829 A (AGRACETUS, INC.) 12 June 1991</p> <p>see column 2, line 8 - column 3, line 10</p> <p>see column 4, line 50 - column 6, line 26</p> <p>see column 16, line 52 - column 17, line 10; claims 7,14,22</p> <p>&amp; US 5 177 308 A</p> <p>cited in the application</p> <p>---</p> <p>-/--</p>	<p>1-6,8, 10-16</p>

☒ Patent family members are listed in annex.

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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11. 03. 1997

Muellners, W

## INTERNATIONAL SEARCH REPORT

International Application No

PL./US 96/06076

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 03588 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA ) 17 February 1994 cited in the application see page 1 - page 3, line 18 ---	7
A	EP 0 505 207 A (THE WELLCOME FOUNDATION LIMITED) 23 September 1992 see page 2 - page 3 see page 6, line 45 - line 50 ---	1-16
A	GENE, vol. 100, - 1991 AMSTERDAM NL, pages 131-7, XP002025567 X WANG ET AL.: "Baculovirus vectors for multiple gene expression and for occluded virus production" cited in the application see page 131, the summary see page 135, left-hand column, line 4 - right-hand column, line 4 see page 136, chapter conclusions ---	1-16
A	BIOCHEMISTRY, vol. 30, 1991, EASTON, PA US, pages 4814-21, XP002025566 E. ZLOTKIN ET AL.: "Functional Duality and Structural Uniqueness of Depressant Insect-Selective Neurotoxins" cited in the application see the whole document see in particular page 4820, paragraphs 2 and 3 and the penultimate paragraph ---	1-16
A	EP 0 477 676 A (BAYER AG) 1 April 1992 see page 2, line 1 - line 21 ---	1-16
A	BIOCHEMISTRY, vol. 31, 1992, EASTON, PA US, pages 7622-8, XP002025569 D. GORDON ET AL. : "Localization of Receptor Sites for Insect-Selective Toxins on Sodium Channels by Site-Directed Antibodies" cited in the application see the whole document see in particular page 7627, the last two paragraphs ---	1-16

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## INTERNATIONAL SEARCH REPORT

International Application No  
PL./US 96/06076

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BIOCHEMISTRY, vol. 29, 1990, EASTON, PA US, pages 5941-7, XP002025568 M. EITAN ET AL.: "A Scoprion Venom Neurotoxin Paralytic to Insects That Affects Sodium Current Inactivation: Purification, Primary Structure, and Mode of Action " cited in the application see page 5941 - page 5942, paragraph 1 see 5946-5947, chapter discussion -----</p>	1-16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/06076

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
Please see attached sheet ./.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 06076

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Claim 9 is not clear. It claims two insect toxins having amino acid sequences, but specifies the sequences as SEQ ID NO:1 or SEQ ID NO:2. The former however is according to the description a nucleic acid sequence. The sequence SEQ ID NO:2 is attributed to the protein toxin LqhIV. A second toxin, designed LqhVI, has instead the amino acid sequence SEQ ID NO:3. It was assumed that claim 9 was intended to refer to the protein sequences SEQ ID NO: 2 and SEQ ID NO:3, but a search for nucleic acid sequence SEQ ID NO:1 was also made.

information on patent family members

PL 1/US 96/06076

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